Volume 194, number 2 FEBS 3229 January 1986

Nonenzymatic radiolabeling of protein by ^{32}P-containing nucleotides

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Received 21 October 1985

We report a nonenzymatic reaction which results in the radiolabeling of proteins by ^{32}P-containing nucleoside triphosphates. The labeling reaction does not require any cofactors, but is greatly enhanced by the presence of alcohols. Even under optimal conditions, less than 1% of the protein molecules undergo modification. This nonspecific labeling represents a serious artifact which may become significant in systems involving low levels of specific labeling, such as photoaffinity labeling. Since the reaction is not limited to specific proteins, this may, however, provide a simple and rapid procedure for the preparation of labeled proteins.

Nonenzymatic protein phosphorylation *Protein radiolabeling* *Photoaffinity labeling* *nusA protein*

1. INTRODUCTION

Numerous examples of post-translational, covalent modifications of proteins have been reported [1]. In vivo modifications of proteins are generally catalyzed by specific enzymes, and many are reversible modifications involved in regulation [2]. Because of the importance of such regulatory modifications, many studies are undertaken in vitro in cell extracts or with purified proteins searching for activities that lead to modifications such as phosphorylation. We have recently discovered a novel reaction which leads to phosphorylation of many proteins in an apparently nonenzymatic reaction, under reaction conditions similar to those used for many biochemical studies. This reaction can pose problems for those studying enzymatic protein modifications, but may also prove useful for the preparation of labeled proteins.

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2. MATERIALS AND METHODS

Bovine serum albumin (BSA) and chicken egg albumin (ovalbumin), purchased from Sigma, were dialyzed against 10 mM EDTA followed by glass-distilled water. The purification of the nusA protein has been described [3]. ^{32}P, purchased from New England Nuclear as carrier-free H_3PO_4, was used to prepare both [α-^{32}P]CTP [4] and [γ-^{32}P]ATP [5] as described; the final specific activities were 1×10^5 and 1×10^7 cpm/pmol, respectively. 2′,3′-Isopropylidenecytidine was purchased from Sigma. [α-^{32}P]dCTP and [α-^{32}P]GTP were purchased from Amersham.

2.1. *Protein labeling*

All reactions were performed in sealed 0.5 ml polypropylene centrifuge tubes. Unless otherwise indicated, reaction mixtures (20 μl) containing 25% (v/v) ethanol, 0.5 μM [α-^{32}P]CTP and 2 μg purified protein were incubated at 37°C for 60 min. 5 vols cold acetone were added and after 15 min on ice, the reaction mixtures were centrifuged for 15 min at $10000 \times g$. Supernatant fractions were removed and the pellets dried prior to being

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00145793/865/$3.50

suspended in 20 μl protein sample buffer [6]. Samples were heated at 90°C for 2 min and the products resolved in a 10% polyacrylamide gel as described [6]. Proteins were stained with Coomassie blue prior to autoradiography.

3. RESULTS

3.1. *Labeling of proteins in vitro by [α-^{32}P]CTP*

In an attempt to perform photochemical cross-linking of purified *E. coli* nusA protein [3] during transcription in the presence of [α-^{32}P]CTP, we observed that the protein became labeled even in the unphotolyzed control reaction. Labeling was initially detected by radiography after electrophoresis of the protein on SDS-polyacrylamide gels. Hence the ^{32}P label appeared to be covalently attached to the protein. We initially interpreted this result as signifying that the nusA protein preparation had an intrinsic kinase or nucleotidyl transferase activity. However several properties of the reaction seem to rule out this possibility. First, labeling occurs even if the proteins are previously denatured by heating to 90°C or by treatment with 0.2% SDS. Second, labeling under these conditions is not unique to the nusA protein (fig.1). Purified preparations of BSA, ovalbumin, *E. coli* RNA polymerase and rabbit triosephosphate isomerase are similarly labeled, and a large number of proteins are labeled in extracts of *E. coli* or HeLa cells (these extracts were incubated at 90°C in 0.2% SDS prior to the addition of label to prevent action of endogenous kinases or nucleotidyl transferases). Purified preparations of several other proteins are similarly labeled. Although there are differences in the efficiency of labeling of particular proteins, all proteins we have tested undergo modification.

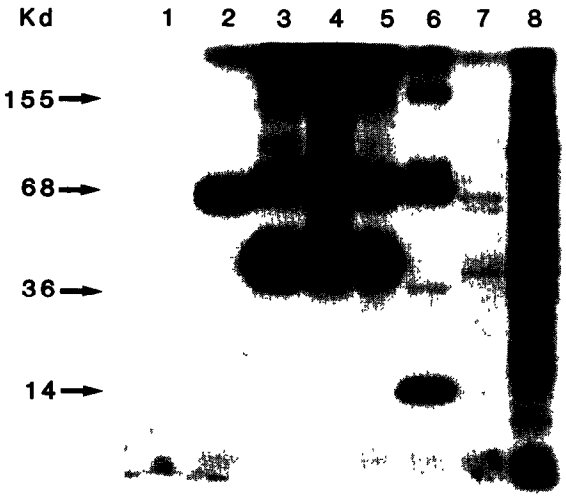

Fig.1. Labeling of proteins in vitro by [α-^{32}P]CTP. Protein labeling was performed as described in section 2. Lanes: 1, no protein; 2, *E. coli* nusA protein; 3, 4 and 5, BSA and ovalbumin; 6, *E. coli* core RNA polymerase, BSA and rabbit triosephosphate isomerase; 7, 25 μg *E. coli* extract; 8, 25 μg HeLa cell extract. BSA and ovalbumin were heated for 5 min at 90°C (lane 4) or treated with 0.2% SDS (lane 5) prior to the addition of [α-^{32}P]CTP.

3.2. *Properties of the reaction*

The extent of incorporation of [α-^{32}P]CTP into protein increases linearly for about 1 h and continues at a reduced rate for at least 20 h (fig.2). However, even after 20 h only a small fraction of the protein has reacted and the molar ratio of CTP incorporated/protein at this time is 0.0048 for BSA and 0.0052 for ovalbumin. All the data are consistent with the phosphate donor being a nucleoside triphosphate. However since the efficiency of labeling is so low, we cannot rule out the possibility that a minor contaminant in the [α-^{32}P]-CTP preparation is the direct phosphate donor. Alternatively, protein labeling activity may be due

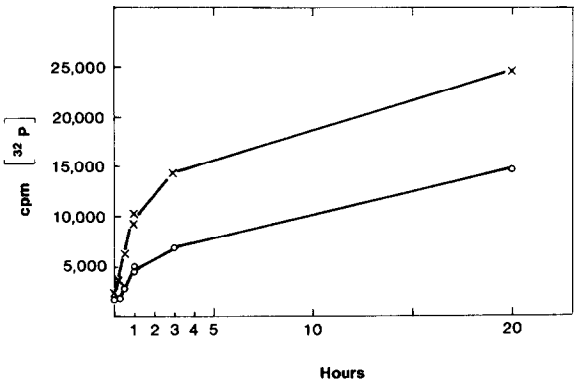

Fig.2. Reaction kinetics. Labeling reactions were performed as described in section 2 except that the incubation time at 37°C was varied as shown on the *x*-axis. Gel slices containing Coomassie-stained proteins were counted by liquid scintillation. (○) BSA, (×) ovalbumin.

to species such as nucleoside tetraphosphates which are present in low concentrations in all nucleoside triphosphate solutions. The protein labeling activity chromatographs with CTP when [α-^{32}P]CTP is isolated on QAE Sephadex as described [4] and excess unlabeled CTP inhibits the reaction by greater than 10-fold (not shown). If a minor contaminant is responsible for the protein labeling activity, then it must be present in nucleoside triphosphate solutions from a variety of sources including [α-^{32}P]CTP synthesized in this laboratory, [α-^{32}P]GTP and [α-^{32}P]dCTP synthesized by Amersham and unlabeled CTP from PL Biochemicals.

The reaction does not seem to require specific cofactors; labeling is observed in distilled water. However, alcohols seem to enhance the reaction (fig.3) and 25% ethanol leads to as much as a 10-fold increase in incorporation after 1 h. Added metal ions were not required and the reaction was not blocked by dialysis of the proteins against 10 mM EDTA. Labeling was observed even at 0°C and was increased at elevated temperature up to 70°C (fig.3).

3.3. *Characterization of the protein-nucleotide bond*

The stability of the protein-[α-^{32}P]CTP adduct in acid and base was tested by soaking acrylamide gel slices in 1 M HCl or 1 M NaOH for 15 h at

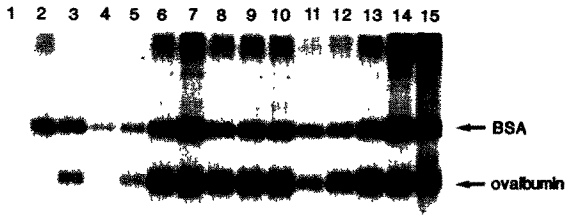

Fig.3. Reaction conditions. Reactions containing BSA and ovalbumin were performed as described in section 2 except as noted. Lanes: 1, no protein added; 2 and 3, no ethanol added; 2, acetone precipitation was omitted; 4, 5, 6 and 7 contained 5, 15, 25 and 50% (v/v) ethanol, respectively; 8, 9 and 10 contained 25% (v/v) methanol, isopropanol or *n*-butanol, respectively, rather than ethanol. All of the above reactions were incubated at 37°C for 60 min. Lanes 11, 12, 13, 14 and 15, incubated for 60 min at 0, 24, 37, 70 and 92°C, respectively.

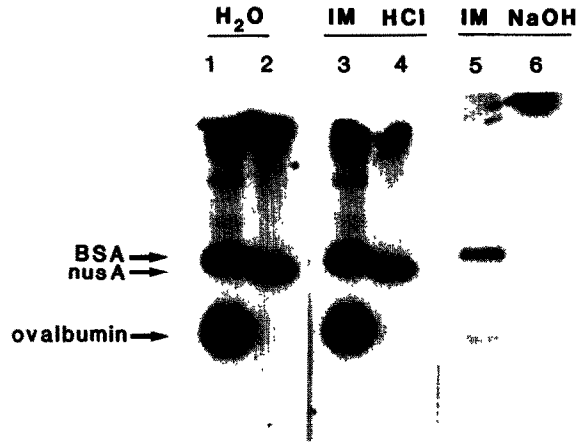

Fig.4. Adduct stability in acid and base. BSA and ovalbumin (lanes 1, 3, and 5) and nusA protein (lanes 2, 4, and 6) were labeled with [α-^{32}P]CTP as described in section 2. Gel slices were soaked with agitation for 15 h at 25°C as indicated prior to autoradiography.

25°C prior to autoradiography. 1 M HCl did not significantly alter the amount of label retained in the gel (fig.4). In contrast, 1 M NaOH reduced the retained label by as much as 90%. It seems likely that the reaction was not a simple phosphorylation or nucleotidyl addition, since [α-^{32}P]CTP, [α-^{32}P]-dCTP, [α-^{32}P]GTP and [γ-^{32}P]ATP are incorporated. Incubation of proteins with [^{32}P]H_3PO_4 did not result in protein labeling (not shown).

4. DISCUSSION

The nonenzymatic labeling of proteins by ^{32}P-labeled nucleotides, which is enhanced in the presence of ethanol, may cause problems for those attempting specific radiolabeling of components with such nucleotides. We first discovered the problem while attempting photoaffinity labeling of specific proteins involved in transcription [7] and found that the nusA protein became radioactive even in the control reaction which was not irradiated. Such labeling was not detected unless the samples were ethanol precipitated. Since ethanol precipitation is a commonly used technique, researchers studying protein modifications should be aware of the potential artifacts created by this nonenzymatic labeling.

The nature of the protein-nucleotide bond has not been determined. We have shown that the ad-

duct is acid-resistant, ruling out the possibility of a phosphoamide linkage, as these bonds are hydrolyzed in acid [8]. The protein-[α-^{32}P]CTP adduct is base-labile, consistent with a phosphoester linkage. This reaction shows no apparent base or sugar specificity since a variety of ^{32}P-labeled nucleotides are incorporated. Both (α-^{32}P)- and (γ-^{32}P)-nucoleotide-labeled proteins, with labeling by (α-^{32}P)-nucleotides being approx. 10-times more efficient.

This method of protein labeling can be put to good use, however. Incubation of specific proteins with ^{32}P-labeled nucleotides in the presence of ethanol provides a simple and rapid method for preparing radioactively labeled proteins.

ACKNOWLEDGEMENTS

We gratefully acknowledge Michael Chamberlin in whose laboratory this work was performed. This investigation was supported by Research Grant GM12010 from the Institute of General Medical Sciences, US Public Health Service, by National Research Award Training Grant GM07232 to M.S., and by California Division, American Cancer Society Junior Fellowship number J-47-83 to M.H.

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